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Scale-down assessment of the sensitivity of *Yarrowia lipolytica* to oxygen transfer and foam management in bioreactors: investigation of the underlying physiological mechanisms

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Abstract A scale-down investigation of the impact of local dissolved oxygen limitation on lipase production by *Y. lipolytica* has been performed. One of the major issues encountered during this kind of process is foam formation, requiring a reduction of the overall oxygen transfer efficiency of the system in order to keep antifoam consumption to a reasonable level. A regulation strategy involving oxygen enrichment of the air flow through the reactor has allowed this issue to be partly overcome. For a second time, the scale dependency of the process operated with air enrichment has been investigated by a combination of scale-down and pilot-scale cultivation tests. The scale-down apparatus considered in this work comprised a well-mixed part connected to a plug-flow part subjected to dissolved oxygen limitation. Surprisingly, foaming intensity was greatly reduced in the case of the test performed in scale-down reactors (SDRs) while maintaining the same stirring and aeration intensities in the stirred part of the reactor. For mean residence time of 100 s in the recycle loop of the reactor, foam formation was significantly reduced while cell growth and lipase production were both unaltered. When the residence time in the recycle loop was raised to 200 s, the foam phenomena was also reduced, but the lipase yield was altered as well as *lip2* gene transcription and translation as shown by real-time quantitative polymerase chain reaction (RT-qPCR) and reporter gene activity, respectively. Our results clearly show the importance of primarily taking into account cell physiology for the scaling-up procedure.

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Introduction

Scaling up bioprocesses is generally difficult considering the progressive loss of mixing efficiency with scale and the complex biological responses that can be exhibited when microorganisms are exposed to the resulting heterogeneities [6, 18]. This is especially true in the case of lipase production by the yeast *Yarrowia lipolytica*. Indeed, this process involves important issues at the level of both the physicochemical system (i.e., the bioreactor) and the biological mechanisms involved in lipase synthesis [8]. The mixing efficiency is indeed critical in order to support the high oxygen demand of the microorganism and to provide efficient dispersion of the hydrophobic substrate that is used as the principal carbon source for the synthesis of lipase (in our case, we use methyloleate as a renewable raw material that is recognized as an inducer for lipase excretion by *Y. lipolytica* [5, 9]. Our previous work has shown a critical relationship between lipase yield and dissolved oxygen level. More specifically, a significant reduction of specific lipase production (i.e., the amount of lipase produced as a function of cell density) has been observed when cells are exposed to oxygen deprivation [13]. While other environmental conditions such as pH and carbon source dispersion seem to have no impact on the level of *lip2* activity, a significant reduction of this activity has been observed in the case of oxygen limitation [14]. The complexity comes from the fact that, in industrial bioreactors, cells are exposed to fluctuating oxygen concentration due to the formation of gradients [22]. Microbial cells can exhibit very different responses involving messenger

RNA (mRNA) species, allosteric control of metabolic enzymes, or synthesis of stress proteins as a function of the amplitude and frequency of these environmental fluctuations [15, 17, 19]. The aim of this work is to clarify which physiological level is affected by these environmental fluctuations. For this purpose, we use a set of two-compartment scale-down bioreactors able to reproduce local exposure of microbial cells to dissolved oxygen limitation. These scale-down reactors (SDRs) have been designed by taking into account the fact that this kind of culture is subjected to severe foaming issues due to the release of a high amount of proteins during the process.

Materials and methods

Strain and culture conditions

Yarrowia lipolytica JMY775 was stored at −80°C in working seed vials (cells in suspension in glycerol solution 40%). The JMY775 strain was obtained by genetic manipulation from a lipase-overproducing strain (LgX64.81) modified with a *lip2-LacZ* reporter gene. Cells were first precultivated in a 250-mL baffled shake flask containing 100 mL medium containing glucose (20 g/L), tryptone N1 (10 g/L; BHA, Belgium), and yeast extract (10 g/L). Following the precultivation step, the culture was transferred to the bioreactor. The cultivation media contained, per liter: Agrimul 28 mL (Cognis, France), tryptone N1 7.5 g, and yeast extract 7.5 g. During the experiments, pH was maintained at 7 ± 0.1 by addition of KOH 6 N or H_3PO_4 6 N, and temperature at 30°C.

Bioreactor operations and scale-down experiments

The laboratory-scale bioreactor tests were performed in Biostat B systems (Sartorius) with working volume of 1.5 L and equipped with Rushton disk turbines with six blades (RDT6, $d = 0.05$ m). Stirrer rate was maintained at 700 rpm during the whole experiment. Foaming was controlled by an antifoam probe placed in the headspace of the reactor at 5 cm from the top of the vessel. Foam level was controlled by addition of polyether antifoam Tego KS911 (Goldschmidt, Germany). When activated, the antifoam control system releases an antifoam pulse of 2.5 mL with an interval time between pulses of 30 s. The dissolved oxygen level was continuously monitored using a dissolved oxygen probe (Mettler Toledo InPro 6800). For the first experiment in the well-mixed reactor, the air flow rate was controlled at 0.7 volume of air per volume of culture per minute (vvm). For the other laboratory-scale experiments, oxygen-enriched air (OEA) was used to cope with the increase of dissolved oxygen consumption. In the case of OEA-operated bioreactors, the total gas flow rate was maintained at 0.7 vvm, but the percentage of oxygen in the total gas flow (v/v) was automatically adjusted between 0% and 30% to keep the DOT above 30%. The scale-down bioreactor used in this work comprises a well-mixed stirred tank reactor (STR) connected to a plug-flow reactor (PFR) where an oxygen-depleted zone is generated (Fig. 1). The DOT at the inlet and at the outlet of the PFR were measured by using a noninvasive optical sensor (Presens). The residence time in the PFR was modulated to study the impact of two ranges of oxygen depletion. First, the residence time inside the PFR was fixed at 200 s (SDR 200 s). Three samples were taken each time along PFR for the experiment to monitor the mRNA level at length corresponding to 0, 125, and 200 s of residence time (see the section relating to the RT-qPCR experiments for more details). Second, the residence time inside the PFR was decreased to 100 s (SDR 100 s). Samples for the determination of the mRNA level were taken at lengths corresponding to 0, 50, and 100 s residence time, respectively. These two SDR configurations allow the reproduction of the classical range of variation of mixing time values expected for industrial bioreactors (mixing time in the range of 50–200 s for an industrial bioreactor running at average dissipated power of 1 kW/m^3 [3, 21]). The pilot plant experiment was performed in a 500-L bioreactor (Biolaffite-France; $D = 0.62$ m) with working volume of 350 L and equipped with two RDT4 Rushton turbines ($d = 0.27$ m). The stirrer rate was maintained at 110 rpm. The air flow rate was controlled at 0.7 volume of air per volume of culture per minute (vvm). A second precultivation step was carried out during 24 h in the bioreactor containing 13 L flask medium. After the precultivation steps, a calculated volume was transferred to the 500-L bioreactor so that the initial cell concentration in 500 L corresponded to the 2-L bioreactor during lag phase. The mixing efficiency of the 500-L bioreactor has been evaluated in a previous study [3]. In the case of the operating conditions considered in this work, this reactor exhibits a mixing time of 24 s.

Cell dry weight determination

Biomass yield was determined after elimination of the fatty fraction present in the cultivation medium. Fifteen milliliters of medium was centrifuged at 10,000 × *g* for 20 min (Avanti J-25I; Beckman, USA). The supernatant was eliminated, and the harvested cells were washed twice with water and detergent 2%. Biomass concentration was estimated on the basis of cell dry weight (CDW). Cells from a culture sample of 15 mL were recovered by filtration under vacuum using hydrophobic polyethersulfone filters (0.45 µm; Pall Life Sciences, USA). The filters were then

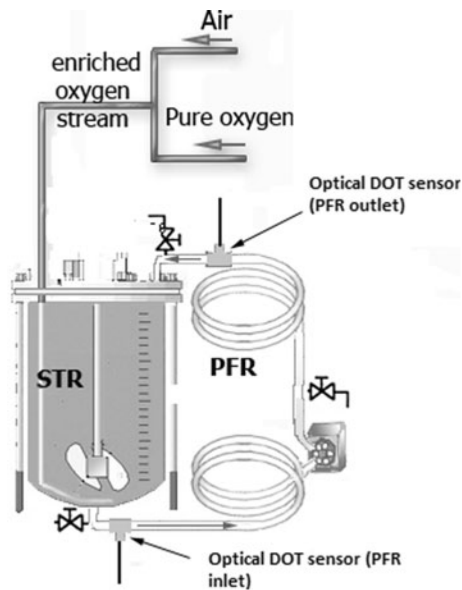

Fig. 1 Scheme of the scale-down reactor (*SDR*) configuration used in this work

placed directly in aluminum dishes and dried at 105°C for 24 h.

Extracellular lipase activity and intracellular *lip2* level of induction

Extracellular lipase activity was determined as follows: samples of culture medium were withdrawn at various fermentation times, and centrifuged for 20 min at 10,000 × *g*. The supernatant was then used for extracellular lipase activity estimation using olive oil emulsion as enzyme substrate [olive oil 25%, 0.1 M NaOH 7.5%, polyvinylic alcohol (2%) 67.5%]. The enzymatic reaction was initiated by adding 1 mL supernatant to 4 mL emulsion with 5 mL 0.1 M phosphate buffer at pH 7. The enzymatic reaction was maintained for 15 min at 37°C on a rotary shaker (150 rpm) and subsequently stopped by addition of 20 mL acetone–ethanol mix [1:1(v/v)]. The free fatty acids released during the reaction were then titrated with 0.05 M NaOH. One unit of lipase activity was defined as the amount of lipase inducing release of 1 mmol fatty acid per minute at 37°C and pH 7. The translation level of lipase mRNA was calculated on the basis of parallel synthesis of β-galactosidase by the LIP2–LacZ reporter gene JMY775 strain. The β-galactosidase activity was expressed in Miller units/mg dry material, where 1 Miller unit is defined as the amount of enzyme releasing 1 μmol *o*-nitrophenol [subsequent to hydrolysis of *ortho*-nitrophenyl-β-galactosidase (ONPG)] per minute and per 1 mg dry material at 37°C. Dry matter was used instead of optical density at 600 nm to measure the β-galactosidase activity to avoid bias attributed to the presence of methyloleate in the samples. β-Galactosidase was extracted from microbial cells using a chloroform permeabilization procedure as described previously [13].

Total carbon and nitrogen sources assay

To determine the concentration of the respective fatty acids present in the broth, 10 mg of each commercial fatty acid, i.e., palmitic acid C16, stearic acid C18, oleic acid 18:1, linoleic acid 18:2, and linolenic acid 18:3 (Sigma–Aldrich), was dissolved in 100 mL acetone for use as internal standards. Gas chromatography analysis was performed using a 5890 series II gas chromatograph from Hewlett-Packard (Palo Alto, CA, USA) equipped with a flame ionization detector and an Alltech AT AQUAWAX column (30 m, 0.25 mm ID, film thickness 0.25 μm). The oven temperature was held at 40°C for 2 min, raised to 165°C at a rate of 1°C/min, raised to 250°C at a rate of 2.5°C/min, and finally fixed at 250°C for 20 min. The injector and detector temperatures were 200°C and 250°C, respectively. The flow of carrier gas (helium) was adjusted to linear velocity of 1 ml/min at pressure of 0.5 bar. One microliter of sample was injected into the gas chromatography (GC) apparatus.

Extraction and purification of total RNA

A culture sample of 15 mL was filtered under vacuum through 0.45-μm-pore-size filters. The filters were placed in a Falcon tube and conserved directly in liquid nitrogen to avoid mRNA degradation. Then, the filters were conserved at −80°C. The E.Z.N.A.™ yeast RNA kit (OMEGA biotek®) was used to extract the RNA. DNase treatment was applied to RNA samples using Turbo DNA-free™ kit (Applied Biosystems) to remove contaminating DNA from RNA preparation. RNA quality and quantification were analyzed using a Genesys™ spectrophotometer. Complementary DNAs (cDNAs) were subsequently synthesized using the High Capacity RNA-to-cDNA kit (Applied Biosystems).

Real-time quantitative PCR

The primers for real-time PCR were designed to have length of about 20–25 bases, G/C content of over 50%, and T_m of about 60°C. The length of the PCR products ranged between 90 and 150 bp. LightCycler™ 163 software (Roche, Mannheim, Germany) was used to select primer sequences. The primer sequences encoding the actin gene were: *ACT-R* (GGCCAGCCATATCGAGTCGCA) and *ACT-F* (TCCAGGCCGTCCTCTCCC) [16]. The primer sequences encoding the Lip2 gene were: *LIP2-R* (ATCTG GTAGTCGGGATACTG) and *LIP2-F* (TTGATCTTGCT

GCTAACATC). All primers were synthesized by Eurogentec (Seraing, Belgium). SYBR green I PCR amplification was performed using a POWER SYBR® GREEN PCR (Applied Biosystems). Amplification was carried out in a 25 μL (final volume) mixture containing 1,000 ng RNA sample, 0.3 μM primer, and 12.5 μL POWER SYBR® GREEN PCR (Applied Biosystems). A negative control without cDNA added was systematically included. Real-time PCR was performed on a StepOne Plus™ real-time PCR system (Applied Biosystems). The experimental design was done using StepOne™ software v2.0.1 installed on a personal computer (PC) connected to the instrument, using the SYBRR green reagents and quantitation-comparative C_T ($\Delta\Delta C_T$) mode. The same program was used to determine the threshold cycle (C_T) values. The amplification procedure involved an incubation step at 95°C for 10 min for initial denaturation, followed by 41 cycles consisting of a denaturation step at 95°C for 15 s, followed by a step at 60°C for 1 min. After the real-time PCR run, melting curve analysis was performed by continuously measuring fluorescence during a temperature upshift from 60°C to 95°C at transition rate of 0.3°C/s. Standard curves were generated by plotting C_T values as a function of log initial RNA concentration. PCR efficiency (E) was then calculated using the following formula: $E = 10 - 1/\text{slope}$. Four dilutions of cDNA were prepared to determine the real-time PCR efficiencies. The actin gene was chosen as a suitable internal control gene to normalize the results. The Pffafl method was used to calculate the fold change in transcript abundance normalized to the actin gene and relative to the sample collected at the beginning of the cultivation process (T1). For each RT-qPCR experiment, statistical analysis was performed on the basis of three independent experiments. Standard deviations were less than 10% of average value. Data of the mRNA analysis are expressed as mean (±standard error of the mean, SEM) of at least three independent samples. Comparisons of different samples were made using one-way analysis of variance (ANOVA) followed by Bonferroni's test using GraphPad Prism (version 4) software. The degree of significance was set at $P > 0.05$ to assess differences between mRNA level.

Results and discussion

Effect of the air oxygen enrichment strategy on lipase production

Generally, foam-related issues arise when the air flow rate is increased to cope with the amount of dissolved oxygen required for microbial growth and synthesis of metabolites. This important consideration leads to a technical solution consisting in reduction of the air flow rate to find a compromise between oxygen requirement and foam formation issues, including injection of large quantities of chemical antifoam agent with the resulting adverse effects. To estimate the physiological impact of this technical solution, two different experiments were carried out in well-mixed, laboratory-scale bioreactors. The first test involved a bioreactor in which severe foaming issues (in terms of the amount of chemical antifoam used) and oxygen limitation are observed. The second test involved a bioreactor under the same operating conditions (stirrer speed and air flow rate) but with progressive oxygen-enriched air (OEA) used to maintain a dissolved oxygen level of 30% during culture. In these conditions, the consumption of chemical antifoam was nearly the same in the two cases (Fig. 2), but with more progressive addition in the case of the bioreactor operated with OEA. This observation is obvious, since the air flow rate is the same for the two experiments. However, the OEA allows the oxygen limitation to be avoided, and the resulting cell growth and lipase production were more elevated than in the case of the bioreactor operated without oxygen enrichment. For the two cases, injection of chemical antifoam began 5 h after inoculation, corresponding to the beginning of the exponential growth phase. This phenomenon can be related to the induction of biosurfactant synthesis and protein leakage by *Y. lipolytica* cells in the presence of hydrophobic substrate. However, the specific lipase production (the ratio between lipase activity and cell density, results not directly shown) is the same in the two cases, indicating that no extreme microbial stress is encountered in our operating conditions. Indeed, our previous work has shown that severe oxygen limitation induces a drop in the level of specific lipase production [13]. To avoid the potential impact of microbial stress resulting from oxygen starvation, the bioreactor operated with OEA will be considered as a reference test in the following experiments.

Scale-down effect on oxygen transfer management and related physiological impact

Four cultivation strategies were considered to investigate the effect of oxygen deficiency and DOT perturbations on physicochemical and physiological response during scale-up. First, culture in a 2-L well-mixed bioreactor was performed with an OEA control; this is used as a reference for the reasons mentioned in the previous paragraph. Second, two scale-down reactor (SDR) tests were performed to investigate stress responses when cells are exposed to local oxygen limitation. Cyclic perturbations in the level of dissolved oxygen level were generated by connecting the stirred bioreactor to a plug-flow reactor (PFR). Scaled-down operating conditions were modulated using the level of the recirculation flow rate between the stirred vessel and the PFR, leading to two distinct mean residence times at the

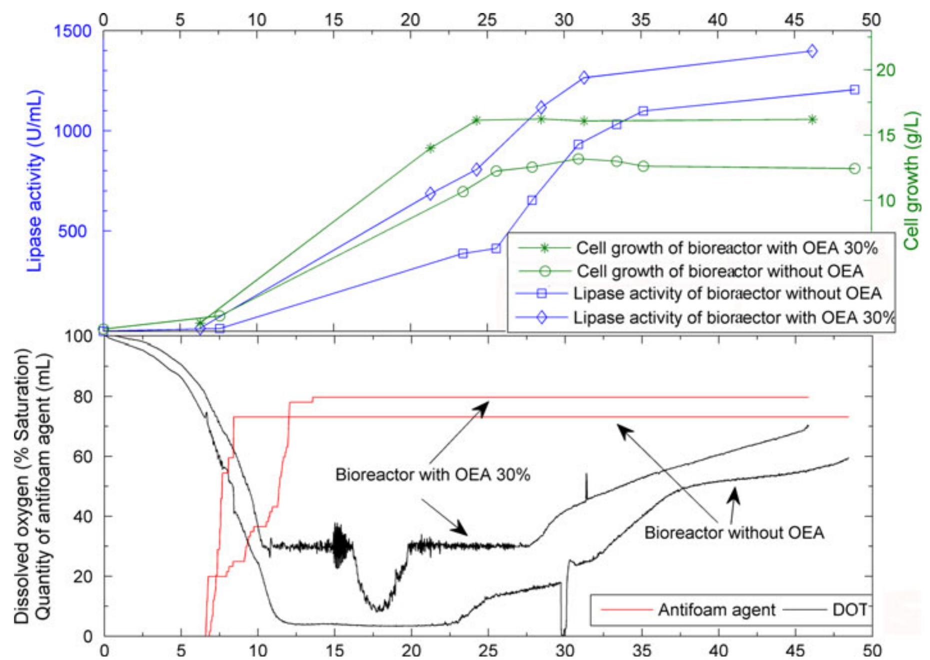

Fig. 2 Impact of the air oxygen enrichment (AOE) strategy on antifoam consumption, cell growth, and lipase production

level of the PFR of 100 and 200 s, respectively. The DOT profiles recorded in the case of the well-mixed reactor and the SDR 100 s were very similar (Fig 3a, b). Indeed, the DOT level remained above 30% with the OEA control, except for a period between 16 and 18 h of culture. This period corresponds to high oxygen demand, as shown by the profile of pure oxygen injection. This phenomenon was not observed in the case of the SDR 200 s (Fig. 3c). The second critical parameter for the process is the consumption of chemical antifoam. Excessive consumption of antifoam agent (80 mL) was observed in the case of the well-mixed reactor, but not in the case of the reactors operated under reduced mixing efficiency, i.e., the SDRs and the pilot plant reactor (5 mL in the case of SDR 100 s, 5.4 mL in the case of the SDR 200 s). This phenomenon can be attributed to the fact that the reduction of the mixing efficiency and the exposure of microbial cells to heterogeneities led to a physiological response that decreased the formation of foam. However, oxygen transfer conditions in the well-mixed reactor and in the SDR 100 s were very similar, as shown by the DOT profiles (Fig. 3a, b). The microbial performance was also very similar, as shown by the lipase yield and biomass growth for these two conditions (Fig. 4b, c). However, when the mixing conditions are altered beyond a given level, i.e., in our case by increasing the residence time in the PFR to 200 s, there is a clear reduction of extracellular lipase activity and cell growth. On the other hand, the intracellular lipase activity (evaluated on the basis of the *lip2 lacZ* reporter system) is similar for all the cultures performed in the laboratory-scale reactor and in SDRs (Fig. 4a). Indeed, our previous work has shown that the impact of stress on *lip2* expression level is only recorded in case of extreme oxygen starvation [13], which is not the case here according to the DOT profiles (Fig. 3a–c). The cell growth and extracellular lipase activity results recorded for the pilot plant reactor are similar to those obtained in the case of the SDR 200 s, i.e., a significant drop in comparison with the well-mixed case and the SDR 100 s. The most interesting result provided by these tests is the significantly lower consumption of chemical antifoam in the SDRs and in the pilot plant reactor. This observation cannot be explained by the lower aeration intensity in the case of the SDR tests. This unexpected result leads to the conclusion that more complex phenomena are involved in the process of lipase production by *Y. lipolytica* and is of primary importance for the scaling-up procedure. Indeed, these procedures are often established by maintaining the same volume of air injected in the reacting vessel [11, 12]. This particular procedure induces an increase of the foaming issues with scale, considering that foam formation is related to the air superficial velocity and not to the air injected per unit of volume (during a classical scaling-up procedure for strict aerobic processes, G/V is maintained constant whereas G/S is proportional to the foam formation intensity, G being the air flow rate in m^3/s, V the reacting volume in m^3, and S the surface area of the reactor in m^2). Thus, on the basis of the chemical engineering rules, scale-up of the lipase production process would lead to an increase of foam-related issues. On the other hand, the scale-down tests have shown that a physiological mechanism leads to a drastic reduction of this kind of issue, as noticed by reduced antifoam consumption. To assess this hypothesis, a test in a 500-L pilot-scale bioreactor was performed and is analyzed in the next section.

Confirmation of the results at the level of a pilot-scale, oxygen-deficient bioreactor

To practically assess the effect observed in scale-down bioreactors, a 500-L pilot plant bioreactor without OEA control was considered to evaluate the effect of scale-up on the physiology of lipase production. The pilot plant reactor test was operated under standard dissipated power (approximately 5 kW/m^3). In these conditions, oxygen transfer is not sufficient, as observed in the DOT profile, which shows oxygen limitation after 6 h and for the rest of the process (Fig. 5). The mixing time value, previously recorded in another work [3], is 24 s under the operating conditions considered. This value can be considered to be very low in comparison with typical values encountered in large-scale bioreactors, but even at this scale dissolved oxygen gradients can be found. The dissolved oxygen probe is located near the wall of the bioreactor, and more elevated values have to be expected in the vicinity of the impellers. If the classical scale-up rules are followed, an increase of foaming issues is to be expected. Indeed, 0.7 vvm air has been considered as a scale-up factor between tests conducted in the 2-L reference bioreactor and the 500-L pilot-scale bioreactor. This leads to a superficial gas velocity (ratio between the air flow rate and the bioreactor cross-sectional area, *G*/*S*) of 0.0088 m/s in the case of the laboratory-scale bioreactor and of 0.0716 m/s in the case of the pilot-scale bioreactor. The air superficial velocity is thus increased by a factor of 10 when the culture is scaled up to volume of 500 L. If it is considered that the rate of foam formation is proportional to the gas superficial velocity, a significant increase of foam formation has to be expected at the 500-L scale. However, only 1,600 mL antifoam was consumed during the culture (results not shown), corresponding to a ratio of 4 mL antifoam per 1 mL reacting medium. In the case of the tests conducted in SDR, this ratio was in the range of 2–3 mL antifoam per 1 mL reacting medium, and 75 mL antifoam per 1 mL medium in the case of the 2-L reference bioreactor. This result confirms the fact that, in a heterogeneous reactor, foaming issues become very limited and are strongly dependent on the microbial physiology. However, a significant reduction of all cultivation parameters, i.e., lipase yield and cell growth, was observed for the pilot plant bioreactor compared with the other operating conditions considered in this work, these observations being linked with more severe dissolved oxygen limitation (Fig. 5). This observation is supported by the response of the *lip2* reporter system, which seems to be affected only in the case of the pilot plant reactor. Since there is practical interest in understanding these phenomena for the reduction of foam formation and process intensification, particular attention will be paid to the dynamics of hydrophobic substrate consumption as well as to the molecular aspects behind lipase induction.

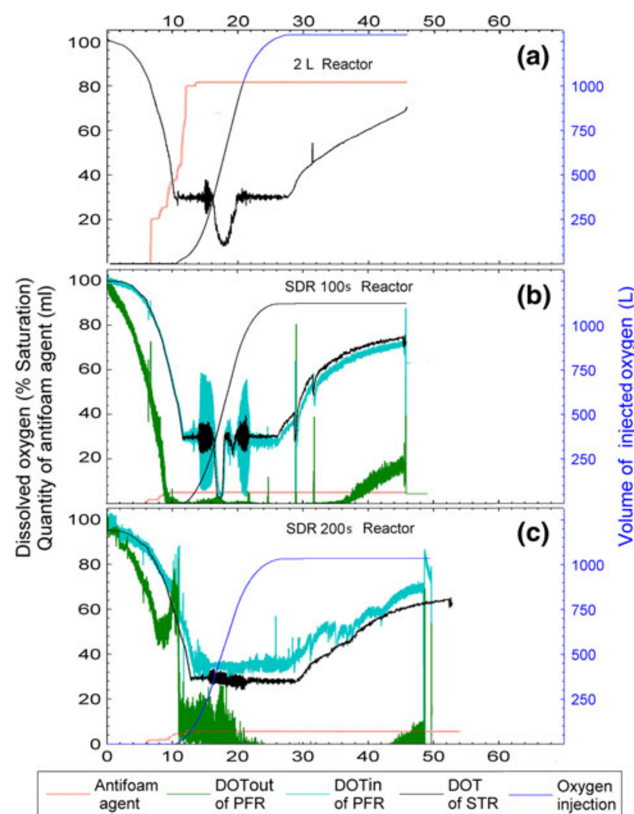

Fig. 3 Dissolved oxygen profiles and antifoam consumption for **a** a well-mixed, laboratory-scale bioreactor, **b** a SDR with mean residence time of 100 s in the recycle loop (DOT profiles at the inlet and outlet of the recycle loop are shown), and **c** a SDR with mean residence time of 200 s in the recycle loop (DOT profiles at the inlet and outlet of the recycle loop are shown)

Impact of process conditions on the consumption of carbon and nitrogen sources

All the cultivation experiments considered in this work were performed by using a low-cost hydrophobic carbon source, i.e., a by-product of the surfactant industry containing mainly methyloleate. This carbon source exhibits a complex composition involving different fatty acids. Since the different fatty acids are removed at distinct rates from the medium and incorporated into the cell [10], this could be an interesting parameter to follow to characterize the impact of scale-down conditions. Fatty acids incorporated inside the yeast cell are either selectively used for energy and as carbon source, or for eventual bioconversion into storage materials [1, 2]. To better understand the scale-down effect on the assimilation of fatty acids, GC analyses were performed at various time points during the cultures (Fig. 6). Considering consumption of fatty acids, it seems that *Y. lipolytica* consumed preferably C18:1, C18:2, and

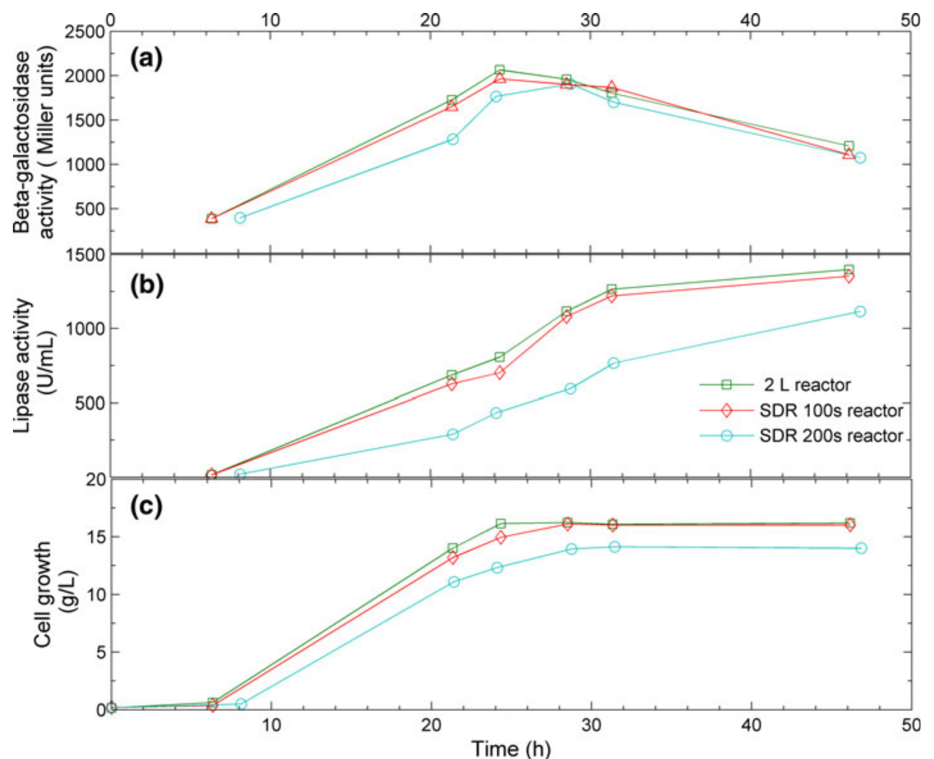

Fig. 4 **a** *lip2:lacZ* intracellular activity, **b** extracellular lipase activity, and **c** microbial growth for the 2-L reference bioreactor, the SDR 100 s, and the SDR 200 s

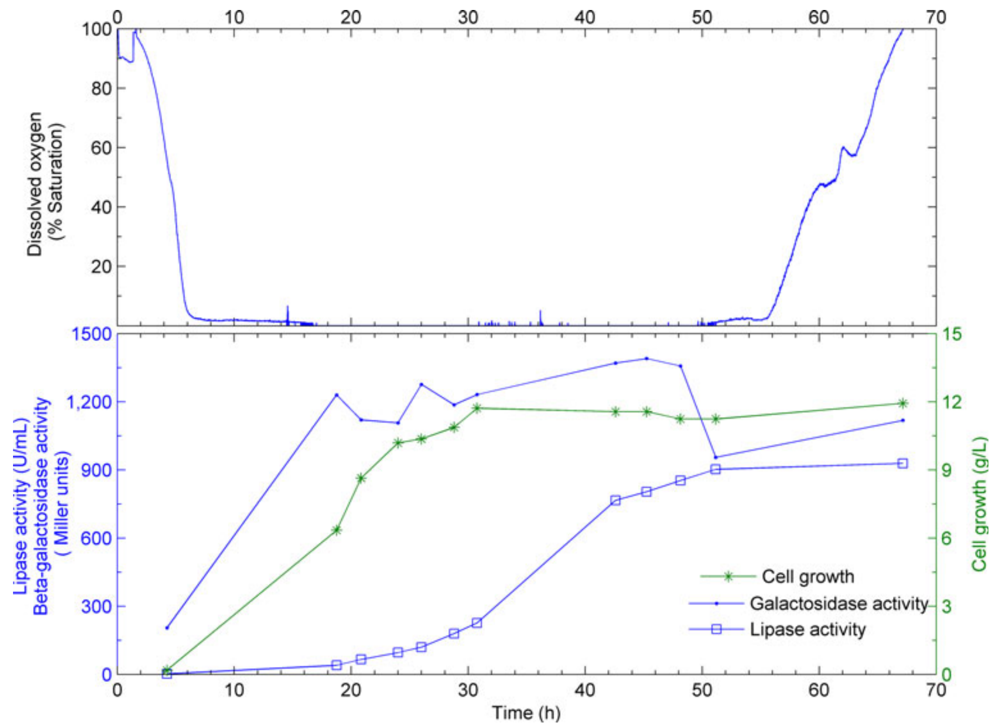

Fig. 5 Evolution of the main cultivation parameters for the test conducted in the 500-L pilot-scale bioreactor

C18:3, with C16 and C18 being consumed to a lesser extent. This observation holds for the experiment performed in both the well-mixed reactor and in SDR 200 s, indicating that selection of fatty acids is not influenced by the mixing performance in these operating conditions. Unsaturated C18 fatty acids are preferentially oxidized for growth requirements. These observations are in accordance with the literature. Unsaturated fatty acids are completely exhausted at the end of culture, while C16 and C18 remain to a large degree in the broth (only 30–40% of these fatty acids are consumed during the process) and cannot be consumed as an energy source by *Y. lipolytica*. On the

other hand, the hydrophobic carbon source is not fully consumed in the case of the culture performed in the pilot plant bioreactor, and a large portion of both saturated and unsaturated fatty acids remains in the broth at the end of the culture (Fig. 6a, d). Nitrogen source could also be another limiting factor. Total nitrogen has been tested, and the results obtained in classical and scale-down bioreactors indicate that around 40% of the initial total nitrogen content was not consumed and remained in the broth at the end of the culture (results not shown). In the case of the cultures performed in the laboratory-scale bioreactor and in the SDRs, the carbon source was completely assimilated at the end of the culture, whereas the nitrogen source was in excess and a significant amount remained in the broth. On the other hand, the carbon source is not completely assimilated in the case of the culture performed in the pilot plant reactor, explaining the lower lipase yield observed in this case. This observation has to be considered in relation to the extreme oxygen limitation encountered in this reactor. The differences of the use of nitrogen and fatty acids during the cultures in the bioreactor could explain the significant drop of foam formation between the well-mixed intensive bioreactor and the bioreactor exhibiting oxygen deficiencies. Indeed, proteins tend to stabilize the foam, whereas fatty acids act as antifoam [4, 7, 20]. Accordingly, in the pilot-scale bioreactor exhibiting strong mixing and oxygen transfer deficiencies, the rate of consumption of fatty acids is very low compared with the test performed in well-mixed conditions.

Investigating another timescale: scale-down effect on *lip2* mRNA expression level

Considering the characteristic time scales involved in the exposure of cells to oxygen deprivation stress in SDRs, the *β-gal* reporter assay is not the best suited one. Indeed, the exposure time on the order of a few minutes in the bioreactors made it difficult to analyze the stress responses at the level of the dynamics of protein synthesis. Indeed, the characteristic time for protein synthesis is within the range of 4–100 min [19], compared with the recirculation time in the PFR of the SDRs which is within the range of 100–200 s. Instead, transcriptional analysis was used to overcome the difficulty associated with the lag time when analyzing the physiological responses at protein or translational level. In a recent work, the influence of DOT fluctuation was characterized on the basis of quantification of levels of mRNA coding for the *lip2* gene [14]. The results obtained clearly showed that the mRNA level is influenced by oxygen deficiency. The qRT-PCR technique was applied for all the bioreactor configurations considered in this work to estimate the mRNA expression level (Fig. 7). The lowest *lip2* mRNA concentration was observed in the case of the pilot plant bioreactor, for which

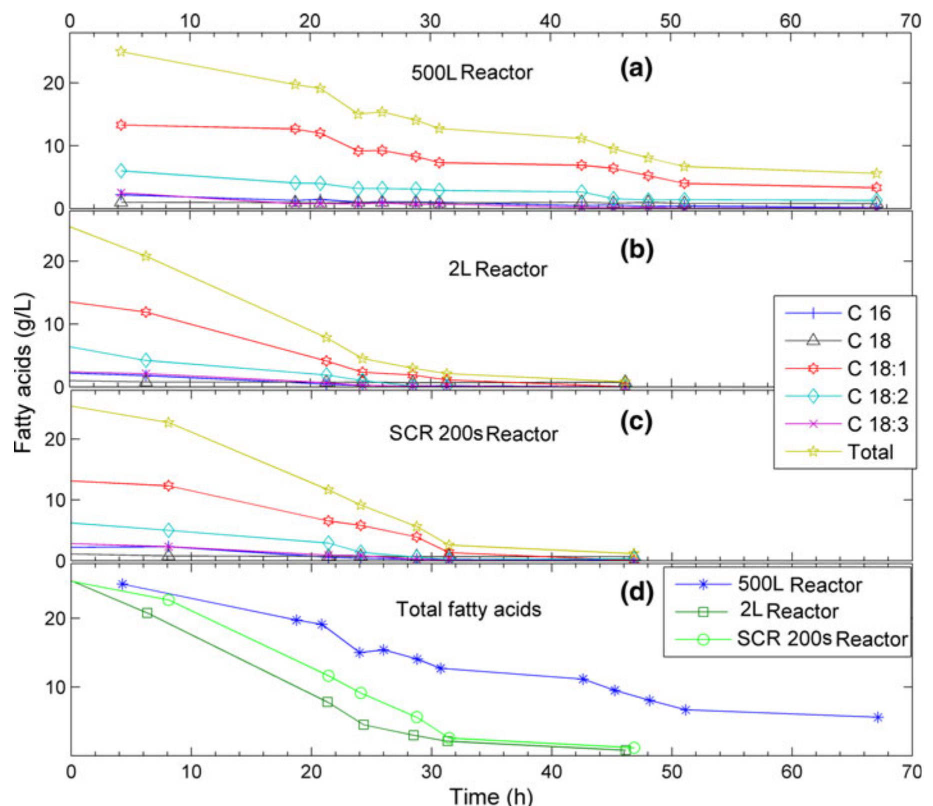

Fig. 6 Evolution of the different fatty acids fractions in different bioreactor configurations. The essential fatty acids of the carbon source used in this work were C16 palmitic acid, c18 stearic acid, 18:1 oleic acid, 18:2 linoleic acid, and 18:3 linolenic acid

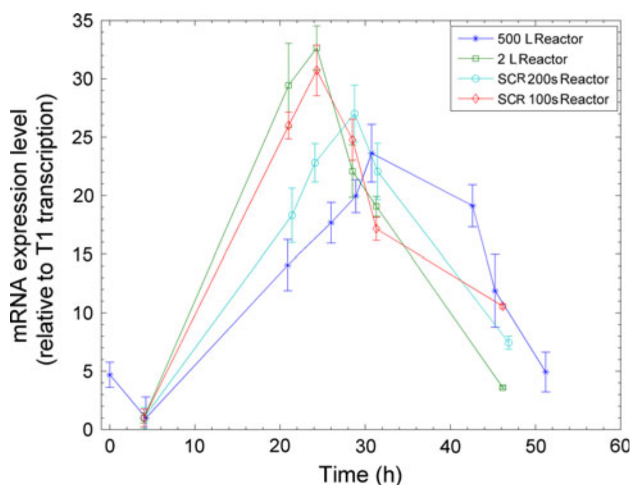

Fig. 7 RT-qPCR profiles of the *lip2* mRNA level for the different bioreactor configurations (numbers of mRNA copies of samples were normalized to T1 at each time)

oxygen limitation is the most intensive. No significant differences were noted for the test conducted in the well-mixed bioreactor or in SDR 100 s. This observation points out that a mean residence time of 100 s inside the recycle loop of the SDR is not sufficient to produce a noticeable stress response at the level of lipase induction. This result is supported by other performance parameters, i.e., cell growth and extracellular lipase (Fig. 2). On the other hand, a drop in the level of *lip2* expression level is observed when the mean residence time is increased to 200 s. Again, this effect is also observed at the level of the macroscopic variables such as cell growth and extracellular lipase activity. The instantaneous *lip2* expression level has also been quantified locally at different levels of the recycle loop of the SDR (Fig. 8). This method allows investigation of the impact of oxygen depletion at given residence time as cells progress through the recycle loop. The concentration of *lip2* mRNA was analyzed in groups of samples at different cultivation periods (21, 24, 28, and 31 h). Samples were withdrawn at the level of three distinct ports along the PFR for each group of analyses, corresponding to residence times of 0, 50, and 100 s for the SDR 100 s and 0, 125, and 200 s for the SDR 200 s. No significant alteration of *lip2* expression was observed for the culture conducted in the SDR 100 s. However, a drop of *lip2* mRNA level was observed for the SDR 200 s in the case of residence times exceeding 125 s. This observation must be correlated to the respective synthesis time of *lip2* mRNA as well as its half-life.

Conclusions

Production of lipase by *Y. lipolytica* is highly dependent on the oxygen availability in the bioreactor, and to a larger extent on the way in which the oxygen transfer is managed as a function of the foam control process. Oxygen air enrichment control has a positive impact on the total amount of lipase produced during the process and allows foaming issues to be dealt with while maintained a sufficient DOT level in the broth. The scale dependency of the OEA-operated process was investigated through a combination of scale-down and pilot-scale cultivation tests. Surprisingly, foaming intensity was greatly reduced in the case of the test performed in SDRs while maintaining the same stirring and aeration intensities in the stirred part of the reactor. For mean residence time of 100 s in the recycle loop of the reactor, foam formation was significantly reduced while cell growth and lipase production were unaltered. When the residence time in the recycle loop was increased to 200 s, the foam phenomena was also reduced, but the lipase yield was altered as well as the *lip2* gene transcription and translation as shown by RT-qPCR and

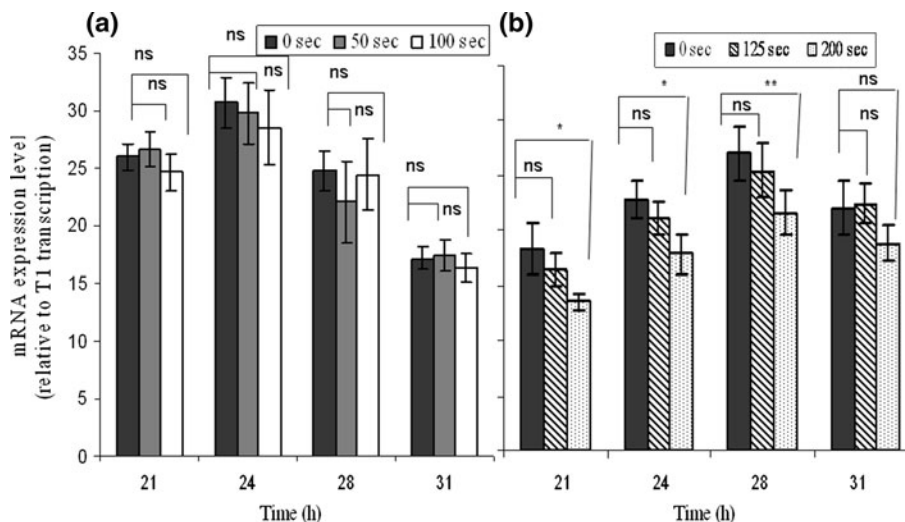

Fig. 8 RT-qPCR profiles of the *lip2* mRNA level at different locations in the SDRs. **a** First series of analyses in PFR for SC 100 sc. **b** Second series for SC 200 sc. The data represent the mean (±standard deviation, SD) of four independent analyses. Comparisons of means were made using one-way ANOVA followed by Bonferroni's test ($*P > 0.05$, $**P > 0.01$, *ns* not significant)

reporter gene activity, respectively. A test performed in a 500-L pilot plant bioreactor exhibiting mixing deficiencies confirmed this result. Our results clearly show the importance of primarily taking into account cell physiology for the scaling-up procedure. The direct physiological parameters, i.e., *lip2* gene expression and translation, affect the global yield of the process, whereas the indirect physiological parameters, i.e., exposure of cells to dissolved oxygen heterogeneities, influence the formation of foam and thus the oxygen transfer management in the reactor. Our results can be exploited to improve oxygen transfer management for lipase production by *Y. lipolytica*. Indeed, cycles involving a short oxygen limitation phase (<100 s) could be implemented to reduce foaming issues while keeping the same lipase yield.

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